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Curriculum

Fitness of Antibiotic-Resistant Bacteria in the Environment: A Laboratory Activity †

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In this laboratory experiment, we propose an opportunity for students to broaden their understanding of the ecology of antibiotic-resistant and sensitive waterborne bacteria. Antibiotics can be found in rivers or soil as a consequence of agricultural practices or as a result of human use. Concentrations of antibiotics in the environment may range from a few ng to µg L-1. Such concentrations can affect the selection and fitness of resistant bacteria. In this laboratory activity, students learn how to set up a fitness experiment by using an isogenic pair of antibiotic-resistant and sensitive bacteria in the presence or absence of selective pressure. Microcosms were generated by using filtered river water containing populations of resistant and sensitive bacteria. Competition of both populations was measured in the presence or absence of antibiotics. Students appreciated the use of microcosms for in vitro experiments and the extent to which the fitness of resistant and sensitive bacteria changed in the presence and/or absence of a selective pressure in river water. Student learning was measured by using different types of assessments: multiple-choice, true/false, fill in the blanks, laboratory skills observations, and laboratory reports. After the laboratory activity, the percentage of correct answers significantly rose from ~20% to ~85%. Laboratory skills were also evaluated during the exercises, showing no major issues during the experiment. Students showed proficiency in analyzing the complexity of fitness data by reaching a mean of 5.57 (standard error 0.57) over a maximum score of 7 points.

INTRODUCTION

Antibiotic resistance poses a serious global health problem due to the overuse and misuse of antibiotics, contributing to the widespread transmission of antibiotic resistance genes in microorganisms (1). Antibiotics are continuously released into the environment from anthropogenic sources such as wastewater treatment plant effluent, hospital and processing plant effluent, application of agricultural waste and bio-solids to fields, and leakage from waste-storage containers and landfills (2). The presence of antibiotics in the environment may range between 0.1 and 1 ng mL^{-1} in rivers to 0.5 µg g^{-1} in biofilms from waste water treatment plants (3–5). Such concentrations potentially can select for antibiotic-resistant bacteria. In this laboratory experiment, we create an opportunity for students to broaden their understanding of the ecology of antibiotic-resistant and sensitive bacteria in water. Acquisition of antibiotic resistance may be associated with a physiological cost for the bacterium, and it brings advantages in case of selective pressure (6). Changes in bacterial fitness may be small and difficult to quantify, and several experimental approaches are currently available (6). In this paper, we present the application of one such method to measure the fitness of antibiotic-resistant bacteria through the generation of microcosms. With this laboratory experiment, both undergraduate and graduate students learn how to measure fitness and observe to what extent antibiotics affect the fitness of bacteria.

In this laboratory experiment, we showed students that resistant and sensitive strains of *Escherichia coli* have a different fitness profile according to the presence of antibiotics in the environment. Students determined the growth rate to measure the fitness costs associated with antibiotic resistance genes in generated microcosms. This three-week laboratory experiment has been developed for students with a focus in environmental microbiology to test how the fitness of resistant and sensitive bacteria differs when grown in river water in the presence of tetracycline. The ecological consequences and health implications of fitness were also discussed with the students.

Intended audience

This activity was designed for undergraduate Microbiology and Biology majors. The activity presented in this

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paper and its extensions is also suitable for more advanced Master Microbiology courses.

Prerequisite student knowledge

Week 1. Concepts relating to bacterial growth, fitness, sterility, use of pipettes, serial dilution, principles and use of a spectrophotometer, and ecology of rivers (which is not strictly required but could help in providing a broader view of the activity).

Week 2. Knowledge of how to work in sterility and about selective media.

Week 3. Concept of logarithms, use of Excel, Student's *t*-test and use of statistical software.

All these concepts were reinforced during the laboratory as part of the regular syllabus. General safety guidelines were discussed in all microbiology labs before performing the experiments.

Learning Time

Week 1. Two hours are required to accomplish the experiments proposed in week 1. Students were asked to prepare the microcosms from the river water and to inoculate *Escherichia coli* strain 1655 (resistant to tetracycline) and *Escherichia coli* strain 12017 (sensitive to tetracycline) in the microcosms. For detailed information please refer to Figure 2 and Appendices 1 and 2.

Week 2. One hour is required to accomplish the task. Students will be focused on performing the experiment to discriminate resistant versus sensitive bacteria (Fig. 2 and Appendices 1 and 2).

Week 3. Two hours are required for this task. Students are asked to perform some mathematics and statistical analysis such as calculating the competition index and running Student's *t*-test (as shown in the student's handout, Appendices 2 and 3). The instructor should use 30 minutes at the beginning of the laboratory activity to refresh mathematical and statistical concepts.

Learning objectives

Upon completion of this three-week laboratory, students will be able to:

1) Demonstrate comprehensive knowledge about the generation of a fitness experiment by using an isogenic pair of antibiotic-resistant and sensitive bacteria in the presence or absence of selective pressure
2) Perform different microbiological tasks for the accomplishment of the fitness experiment
3) Analyze data with graphical and statistical methods

PROCEDURE

Materials

To accomplish the three-week laboratory, each student required the following materials (a complete and detailed list is available in Appendix 1):

- LB agar plates without tetracycline and 10 µg/mL tetracycline
- *Escherichia coli* MG1655 (wild type) and CAG12017 (tetracycline-resistant strain). The strains can be easily purchased at the *E. coli* Genetic Stock Center (cgsc.biology.yale.edu).
- Filtration kit and filters
- River water
- Gloves and other personal protective equipment
- Sterile toothpicks
- Template with 50 squares

The following equipment was also required: a safety biological hood (BSL level 2) and incubators at 30°C (for the incubation of the microcosms) and 37°C (for the incubation of the LB plates).

Student instructions

Safety rules were explained to the students. After the introduction, students received a laboratory handout with a table to register their findings (please refer to Appendices 2 and 3 for a complete description).

Students were asked to formulate their own hypothesis about the fate of resistant and sensitive bacterial strains under selective pressure. In the handout, examples of hypotheses were provided (Appendix 2).

Students worked alone or in pairs. Each pair (or single student) was responsible for the generation of their own microcosms (Fig. 1). The microcosms were incubated for 2 days at 30°C to allow *E. coli* to replicate.

During the second week, students were instructed on how to transfer colonies from the non-selective LB plates to plates with a selective antibiotic, in this case tetracycline. Once the concepts were understood, students started the transfer (called "patching") onto new plates using sterile toothpicks. Students patched 50 colonies from each plate and the 'patched' plates were further incubated overnight at 37°C. It was important that students used a sterile toothpick for each transfer.

During the third week, students counted patches that grew and those that did not on selective media, reflecting the percentage of resistant and sensitive bacteria (Fig. 2), and reported the results on their cards (Appendix 3). At the end of the data collection, all cards were collected by the instructor and tabulated into a spreadsheet and the data were projected onto a whiteboard for group analysis and discussion. The Log_{10} (Competition Index) [Log(CI)]

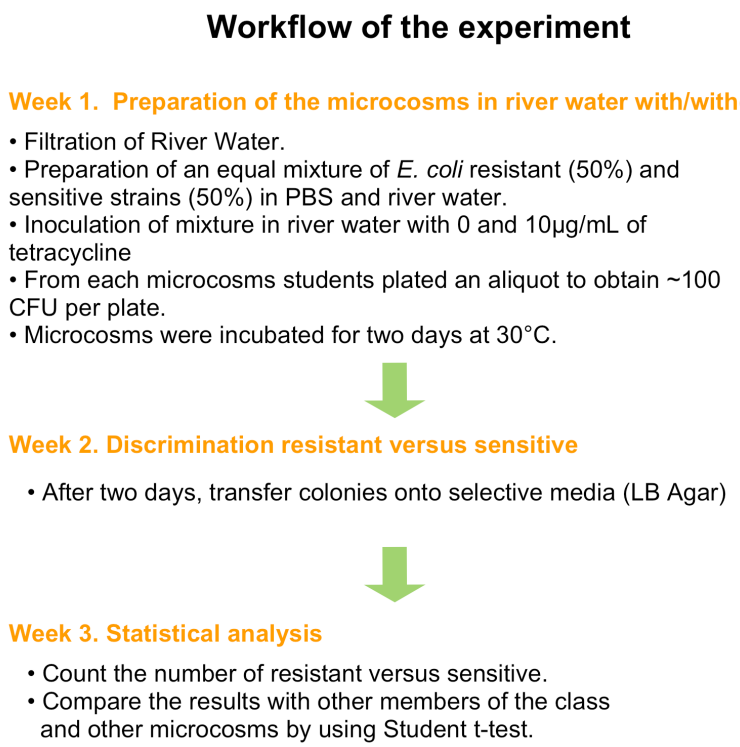

FIGURE 1. Example of workflow of the experiments conducted over the three-week period. PBS = phosphate-buffered saline.

was calculated according to the equation in the student's handout. The spreadsheet was opened with the software JMP (SAS) and the analysis was performed in front of the students. Finally, the spreadsheet was uploaded onto the online learning management system to be accessible to all the students. The instructor encouraged the students to replicate the analysis by using the spreadsheet online and the statistical software downloadable from the University's website. Students may also be asked to write a laboratory report about their findings.

Faculty instructions

The most time-consuming activities of this experiment are the preparation of LB plates, which requires three hours, and the collection of river water. All preparatory activities can be carried out one or two days before the first week. Any further preparatory work needed during the experiment requires one to two hours. Specific faculty instructions are detailed in Appendix 1.

Approximately two hours were required to accomplish experiments in the first week. The instructor should pass through the benches to observe the students' progress, ensuring adherence to safety procedures and addressing questions and concerns. Generation of microcosms was the most complex part of the process. Instructors should also consider preparing mixtures of resistant and sensitive bacteria in front of the class and provide the students the same mixture for the initial inoculum of the microcosms. This strategy has the advantage of having students all start with the same number of CFU/mL. The second week mainly involves patching the colonies onto selective media and should only require one hour to accomplish. The instructor should prepare the plates with tetracycline (or the appropriate antibiotic) before the laboratory experiment. We arranged several copies of a plate template with a 50-square grid to facilitate the transfer of colonies. During the third week, two hours were required to complete counting, summarize the results in the reporting card, and carry out collective statistical analysis of the data (Fig. 2).

Instructors may use any statistical software or methodology that they expect to be more favorable. However, we would strongly recommend that the instructor run Student's *t*-test to determine whether the results reveal any statistically significant differences in cell counts among the samples. A typical result is shown in Figure 2. At first, students were instructed how to read the values from Log(CI). The Log(CI) allows numerical measurement of the replicative ability relative to a pair of competing isogenic organisms in a particular environment. The Log(CI) is identified by either a negative number, positive number, or 0. When values are negative, the sensitive strains are proliferating faster; when values are above 0 the resistant strains are proliferating faster. If the value is Log(CI)=0±0.2, both the wild type and the mutant strains are replicating at the same rate. A spreadsheet with the calculation is available in the Supplemental Materials (with the example reported in the Student card, Appendix 3). To focus on the hypothesis and current knowledge in

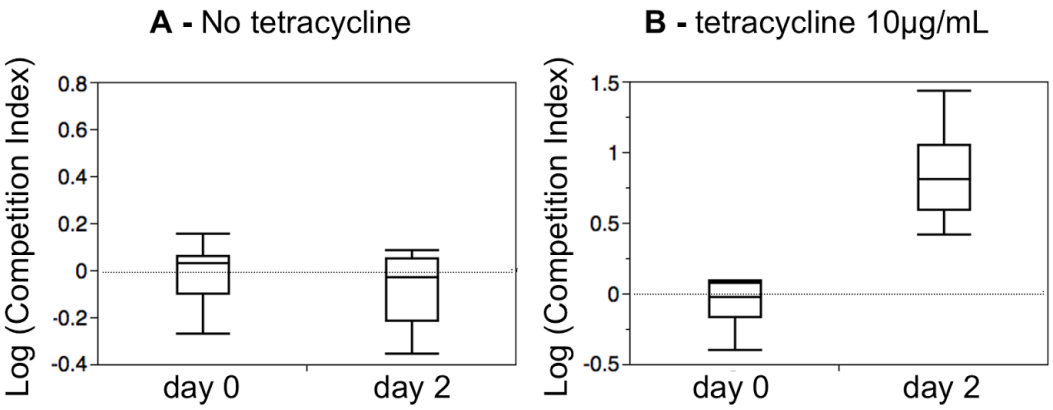

FIGURE 2. Main findings reported after the three-week laboratory experiment. Box plots represent the fitness of resistant and sensitive bacteria under different concentrations of tetracycline. On the *y* axis, negative value represents sensitive bacteria that are out-competing the resistant bacteria. Positive values represent resistant bacteria outcompeting the sensitive cells. When the ratio is equal the competition index is 0. (A) the fitness of resistant and sensitive bacteria at day 0 and day 2 in the absence of tetracycline. Microcosms were prepared using Thames River water in which the microbial community was removed via filtration. (B) In the presence of tetracycline (selective pressure), resistant bacteria have an increased fitness. Boxes include the lower and upper quartiles, lines within the box are the medians and whiskers indicate the degree of dispersion of the data.

detail, the references for three recent papers about fitness and selective pressure of antibiotic resistant bacteria were provided at the end of the student handout (Appendix 2). For example, the paper by Deng and coworkers (7) reports the occurrence and risk assessment of antibiotics in river water in Hong Kong. We discussed the occurrence of antibiotic resistance in the river of our city, London, and the risk for replication of outcompeting resistant bacteria. Other interesting work that was also discussed with the students was an article by Amos investigating how the occurrence of variables pertaining to resistance levels from different sampling sites (proximity, size, and type of surrounding wastewater-treatment plants) affected the occurrence of antibiotic resistance (8). Students were also taught that most antibiotic-resistance mechanisms are associated with a fitness cost that could be observed as a reduced bacterial growth rate in the absence of antibiotics. On the other hand, when the antibiotic is present, the environmental pressure selects resistant bacteria (9). In this experiment, students appreciated that in the presence of the antibiotic, the fitness of resistant bacteria was significantly improved when compared with the control at day 2 (Fig. 2B).

Finally, students were asked to write a laboratory report about their findings. The laboratory report may be formative or summative, graded, and included in the students' final examination mark. The student handout (Appendix 2) can be attached to the laboratory report. The report should include an Introduction, Materials and Methods, Results, and Discussion, as well as an additional Bibliography section showing students' further readings. Open-ended questions at the end of the student handout were discussed with students during the laboratory activity.

Suggestion for determining student learning

We strongly encourage the use of a student handout (Appendix 2). Student handouts should be provided in advance for students to familiarize themselves with the materials and the workflow of the laboratory. Questions to demonstrate comprehensive knowledge about the generation of the fitness experiment are proposed in Appendix 4. A laboratory report should be used as an assessment method to measure the students' learning. Rubrics for these assessments are available in Appendix 5.

Sample data

In Figure 2 we report a typical result, including a dot plot. In Appendix 3, we have included an example of a completed student's reporting card and use of the equation to calculate the competitive index.

Safety issues

This laboratory activity should take place at the end of the semester (or year), in order for students to have demonstrated competency with BSL1 safety procedures before working on this BSL2 activity. The strains used in this laboratory are an isogenic pair of *E. coli* MG1655 (F-, λ-, rph-1) as the wild type and CAG12017 (F-, zaj-3054::Tn10, λ-, rph-1) and with a Tn10 containing *tetRA* genes as the mutant. This isogenic pair was purchased at the *E. coli* Genetic Stock Center (cgsc.biology.yale.edu). For safety reasons, we consider these *E. coli* strains as BSL2 (or Risk Group 2 in the BMBL & NIH classification), considering that the

mutant has tetracycline resistance. To that end, laboratory procedures and/or practices outlined in the submission and in the laboratory must adhere to ASM Guidelines for Biosafety in Teaching Laboratories (10).

Students wore standard laboratory protection (lab coat, closed-toed shoes, and gloves at all times), including working in a BSL2, BSC Type 2 cabinet. Contaminated waste was autoclaved and disposed of according to the Institution's policy and regulations. Instructors or technicians must be in charge of autoclaving the biohazardous waste. At the beginning and end of the experiments, students were instructed on the disposal procedures of the biohazardous material.

To improve safety, and in order to avoid a highly variable unknown sample, river water was pre-filtered before giving it to the students. Students can filter the water again for educational experience.

DISCUSSION

Field-testing and evidence of student learning

Data were collected from seven undergraduates enrolled in the Module "Dissertation" and fifteen undergraduates enrolled in "Environmental and Health Stressors – Microbiology section." The microbiology laboratory sections were organized in these courses during the spring semester in 2016. To evaluate the learning gains, students were assessed using different assessment strategies: pre- and post- tests (LO 1), skills observation (LO 2), and ability to analyze the data (LO 3).

We measured the extent to which students demonstrated comprehensive knowledge about generating the fitness experiment (LO 1). Tests were developed in three different formats: multiple choice, true/false, and fill in the blanks (Appendix 4). Questions varied from general microbiology knowledge to statistics, including understanding of the equation proposed in the laboratory experiment. Results showed that students' knowledge gains were significant after the three weeks (Fig. 3). Interestingly, the percentage of correct answers to the fill-in-the-blank questions was very low in the pre-test, showing the difficulty of the topic taught. After the laboratory experiment, the percentage of correct answers rose significantly, from ~12% to ~85%. Similar results were obtained with the other type of questions. Correct answers to both the multiple choice and true/false questions increased from ~38% on the pre-test to ~85% on the post-test. The percentage of correct answers on the multiple choice and true/false pre-tests was higher due to the fact that students had the opportunity to guess, which was not possible in the open questions.

When proficiency in accomplishing different tasks during the laboratory exercises was measured (LO 2), we observed a good level in performing the experiment (Fig. 4, rubric Appendix 5) (11, 12). No statistically significant differences amongst the means was found (significant probabilities at 0.05%), revealing that no major difficulties were detected while conducting the experiments. Minor differences were identified for the pipetting skills. Students tend to pipette very fast, pressing the volume button multiple times (Fig. 4). We corrected this behavior in the laboratory, but an additional pipetting-skills laboratory should be implemented in any undergraduate course.

Finally, the ability to critically analyze the laboratory results was measured by assessing students' understanding of the results and their ability to integrate them with recent literature. Discussion sections of the laboratory reports were evaluated using the rubric proposed in Appendix 5 (LO 3). We assigned a score between 0 and 7, obtaining a mean score of 5.57 (standard error 0.57). The response to this task overall was very good, showing not only a good understanding of the laboratory activity but also a good integration of data in the discussion.

Students' evaluation of the laboratory activities

At the end of the three weeks, students were asked to evaluate their experience (Table 1). All the students agreed

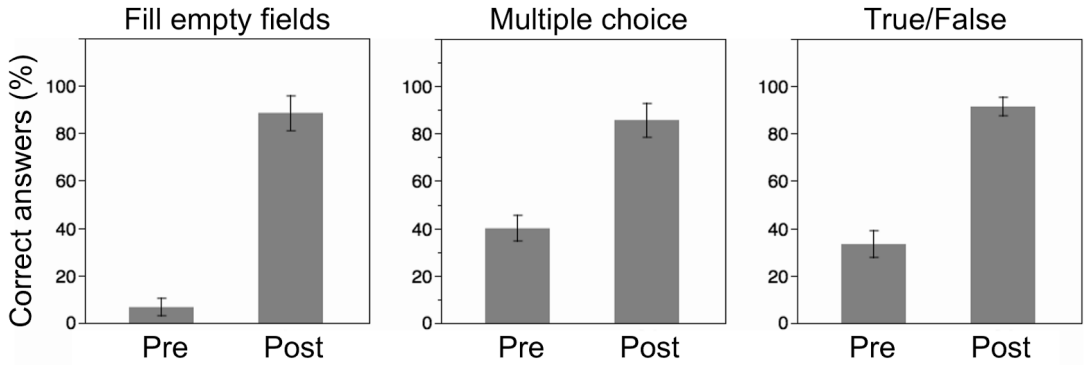

FIGURE 3. Pre-post test assessment LO 1. Percentages of correct answers are shown. The percentage of correct answers was significantly different in all the tests (*t*-test p=0.05). Error bars represent standard error. Please refer to Appendix 4 for the typology of questions.

that the laboratory experiment was well designed and easy to understand.

Possible modifications

The laboratory experiment can be shortened to two weeks by providing the students ready microcosms on week 1 and counting the results on week 2.

Another possible modification is to use resistant bacteria that, in the absence of selective pressure, are less fit than the sensitive bacteria. Unfortunately we did not have strains with this feature. However, it should not be hard to identify some strains with such characteristics. It is well known that the acquisition of antibiotic resistance may be associated with a physiological cost for the bacterium that creates a disadvantage in the absence of the selective pressure (6). The instructor can propose experiments of fitness by changing the environments or antibiotic concentrations.

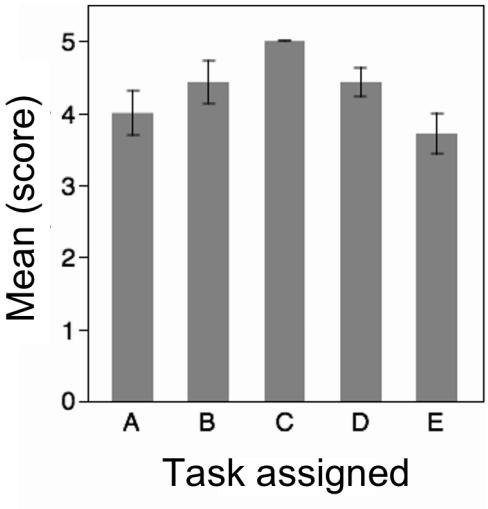

FIGURE 4. Assessment of student performance (LO 2). The scale ranges from very good (5 pts.) to poor (1 pt.). Tasks assigned: A = The student is able to identify contamination on the plates. B = The student can properly patch colonies. C = The student can choose when to use selective plates and non-selective plates. D = The student is able to record the results in the reporting card. E = The student is able to pipette properly.

SUPPLEMENTAL MATERIALS

Appendix 1: Material for the instructor. Procedures for media preparation, inoculation of the strain, collection of river water, consumables and equipment required
Appendix 2: Student laboratory handout and answer key
Appendix 3: Reporting card and example of results
Appendix 4: Pre- and post-activity assessment and answer key to assess LO 1
Appendix 5: Rubrics used to assess LO 2 and LO 3

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TABLE 1.
Students' evaluation of the laboratory activities.

Question	Disagree 1	2	Neutral 3	4	Agree 5[a]
1. I enjoyed the exercises	•	•	•	•	100%
2. The experiments and the materials were well explained	•	•	•	•	100%
3. The experiments and the materials were well organized	•	•	•	•	100%
4. The experiments were easy to perform	•	•	•	•	100%
5. The experiments were well designed to improve my knowledge in studying the fitness of bacteria in the environment	•	•	•	•	100%
6. I know how to discriminate resistant versus sensitive bacteria on selective media	•	•	•	•	100%

[a] These percentages are averaged from the cards recollected from seven students.

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